20, 22-23 and 27-31 have been canceled, without prejudice to refiling. Claims 1, 8, 14, 15, 16, 21, 24, 25 and 26 have been amended.

Attached hereto is a marked-up version of the changes made to the specification and claims by the current amendment. The attached is captioned **"VERSION WITH MARKINGS TO SHOW CHANGES MADE."**

No new matter is added.

Support for the amending language "a composite transcriptional regulatory element" may be found in the specification on page 26, lines 5-6. The inclusion in the composite TRE of a promoter and/or enhancer is provided for in the specification on page 26, lines 15-16. The growth of cells under hypoxic conditions is supported on page 29, lines 1-4.

Support for the amending language, "a binding site for hypoxia inducible factor-1" may be found in the specification on page 22, lines 24-26. As stated in the subject specification, the adenovirus vector CN515 was constructed by inserting a 67 base pair fragment from HRE eno1 (Jiang *et al.* (1997) *Cancer Research* 57:5328-5335) into CN65 at the BglII site (page 49, lines 14-16) As discussed by Jiang *et al.*, increased expression in hypoxic cells is mediated in part by binding of HIF-1 to cis-acting HREs located primarily in the 5' regions of these genes (p. 5328). The paper goes on to describe (p. 5331) the use of a plasmid construct which contained a 68 bp HRE from the ENO1 gene 5' flanking region. It can be seen in Jiang *et al.* that an Eno1 HRE has the sequence starting with a 5' AGGGCCGGACGTGGGGCCCC, followed by an undefined 28 nucleotides, then the 3' sequence. Jiang *et al.* cite an earlier publication (Semenza *et al.* (1996) J. Biol. Chem **271**:32529-32537) for further details about this HRE. The Semenza *et al.* paper discloses the sequence of a functional HIF-1 binding site in an HRE as "ACGCTGAGTGCGTGCGGGACTCGGAGTACGTGACGGAGCCCC".

The rejection of Claims 10 and 22 under 35 U.S.C. 101 is made moot by the cancellation of the claims.

Claims 1-31 have been rejected under 35 U.S.C. 112 first and second paragraph.

The rejection of claims 1, 8, 9, 11, 13, 18, 21 and 26-31 as indefinite in the recitation of the term "cell status specific transcriptional regulatory element" is made moot by the amendment or cancellation of the claims. Other rejections applied to cancelled claims will not be further discussed.

Claims 9 and 21 have been rejected, stating that the recitation of the term "hypoxia responsive element" was indefinite, and the scope of the claim was not enabled by the disclosure.

The claims have been amended to clarify the nature of the HRE, which is a transcriptional regulatory element comprising a binding site for the hypoxia inducible factor-1 (HIF-1).

The specification discusses, on page 22, line 24 ff, the transcriptional complex HIF-1, which is induced under hypoxic conditions, and which then interacts with binding sites to regulate transcription of genes, including vascular endothelial growth factor, and glycolytic enzymes, including enolase-1. Examples of such hypoxia responsive elements were known and used in the art at the time of the present invention, and are readily accessible to those of skill in the art.

It was known in the art at the time of filing that the binding site for HIF-1 is a 32-base pair hypoxia-responsive element was identified, which contained two hypoxia-inducible factor-1 (HIF-1) binding sites (HBSs). As discussed above, the cited document Jiang *et al.* provides for the eno-1 HRE, including the sequence of HIF-1 binding sites (see legend to Figure 6B), which are 5'-GACGTGGG-3', and 5'-TACGTGAC-3'. One of skill in the art would readily be able to determine the metes and bounds of the claimed invention.

Claim 14 and dependent claims has been rejected as indefinite and in lacking enablement in the recitation of the term "cell type specific TRE". Applicants respectfully submit that one of skill in the art would readily understand the meaning of this term. As set forth in the specification, a cell type-specific TRE is preferentially functional in a specific type of cell relative to other types of cells. In contrast to cell status, "cell type" is a reflection of a differentiation state of a cell which is irreversible.

The specification further provides a number of specific examples of cell-type specific TREs, such as the regulatory elements from prostate specific antigen, alpha-fetoprotein, glandular kallikrein-1 and urokinase plasminogen activator. One of skill in the art would readily select from among the many known cell type specific transcriptional regulatory elements, for use in the present invention.

The antecedent basis in Claim 16 has been corrected.

Claims 21 and 22 have been amended. The relationship between the prostate specific enhancer and promoter and the sequences set forth in SEQ ID NO:3 has been clarified. The positioning of these elements is discussed in the specification on page 25, line 24 to page 26, line 12. The HRE and the prostate specific promoter and enhancer are juxtaposed to form a composite transcriptional regulatory element, which act to control transcription of an operably linked gene.

Claim 25 has been amended to clarify that an isolated host cell in intended.

Claims 26-28 have been rejected as lacking a clear structural nexus between the recited vectors and the phrase "allowing a cell status-specific TRE to function". The claims have been amended to clarify the relationship, and to recite the growth of cells under hypoxic conditions *in vitro*.

The claims have been rejected as lacking an adequate written description for cell status-specific and/or cell type-specific TREs. As set forth above, the specification provides description for HRE, as an exemplary cell status-specific TRE, and for cell type-specific TREs, consistent with the current claims.

The claims have been rejected as lacking enablement for the genus of genes essential for replication. Applicants have specifically recited the adenovirus early genes E1A, E1B and E4 in the presently pending claims. The sequences of these genes and the essentiality for adenovirus replication are known in the art.

Applicants respectfully submit that one of skill in the art would reasonably expect the claimed adenovirus vectors, comprising an adenovirus gene essential for regulation operably linked to a hypoxia responsive element, to provide preferential adenovirus replication in hypoxic conditions. The examples and guidance provided by the application are further supported by work in the field following Applicants' invention.

For example, Ruan *et al.* (2001) Neoplasia **3**(3):255-63 (copy enclosed) describes a hypoxia-regulated adeno-associated virus vector for cancer-specific gene therapy. The presence of a hypoxia responsive element activated through the transcriptional complex hypoxia-inducible factor 1 (HIF-1) in an adeno-associated virus vector yielded a significant increase in gene expression under hypoxic culture conditions.

Similarly, Ido *et al.* (2001) Cancer Research **61**:3016-3021 (copy enclosed) report that a hybrid promoter comprising a hypoxia responsive element in combination with a cell type specific transcriptional regulatory element, (the -fetoprotein promoter). In a retroviral vector, such a hybrid promoter provided for specific cytotoxicity on exposure to hypoxic conditions. This vector also induces cell death when expressed in a tumor xenograft model. These results indicated that the hypoxia-inducible enhancer provides selective and enhanced gene activity.

The post-filing articles confirm the teachings of the present application. It is reasonable to expect that the selective replication of adenovirus set forth in the present application could be practiced by one of skill in the art without undue experimentation. Withdrawal of the rejection is requested.

Claims 1-9, 11-21 and 23-31 have been rejected under 35 U.S.C. 103(a) as unpatentable over either one of Henderson *et al.*, in view of Hallenbeck *et al.*, Walther *et al.*, Dachs *et al.*, Advani *et al.* and Parr *et al.*

Henderson (WO 97/01358) in relevant part teaches conditionally replication competent adenovirus vectors for the transduction of restricted cell types, wherein one or more of the promoters of the early and/or late genes essential for propagation is replaced with a cell specific transcriptional initiation region. Consistent with the definition set forth above, the PSA TRE of Henderson is a cell type specific TRE. The Examiner acknowledges that Henderson does not teach cell status-specific TREs. Accordingly, Henderson does not teach an adenovirus vector comprising an HRE, as encompassed by the present claims.

Hallenbeck (WO 96/17053) teaches replication conditional adenovirus designed to limit replication to specific cell types due to linking an adenoviral early gene to any one of a number of different tissue or tumor-specific promoters. The Examiner acknowledges that Hallenbeck does not teach cell status TREs. Accordingly, Hallenbeck does not teach an adenovirus vector comprising an HRE, as encompassed by the present claims.

Walther et al., 1996, is a gene therapy review directed to targeted vectors for gene therapy of cancer. Walther et al. discloses several types of cell status TREs, which the Examiner states "serve as a source for suitable promoters to be exploited for expression regulation of therapeutic genes" and "therapy-inducible" vectors for expression of auxiliary therapeutic genes. Different from the expression regulation gene therapy vectors described by Walther et al., which by their nature are replication -defective, the present claims encompass replication competent adenovirus vectors which comprise an HRE for cell specific adenoviral gene expression, not transgene expression. The Examiner acknowledges that Walther et al. does not teach or recite "hypoxia-inducible response elements" as encompassed by the present claims.

Dachs et al., Nature Medicine, 1997 teaches targeted gene therapy for treating cancer patients where the hypoxic environment of a tumor facilitates heterologous gene expression. More specifically, the reference describes gene expression in a hypoxic environment which is driven by the mouse PGK-1 HRE fused to the PGK-1 promoter. The Examiner acknowledges that Dachs et al. does not teach or suggest replication competent adenovirus vectors comprising cell-status TREs, as encompassed by the present claims. In addition, Dachs et al. does not teach or suggest replication competent adenovirus vectors comprising an HRE, as encompassed by the present claims.

Dachs et al., Oncology Research, 1997, is a review directed to vectors for targeted delivery of therapeutic genes for gene therapy of caner, which recites numerous cancer-associated regulatory elements. Dachs et al. does not teach or suggest vectors for cell specific adenoviral gene expression.

Advani et al., 1997 is cited as teaching cell status TREs comprising radiation-inducible promoters. The Examiner acknowledges that Advani et al. does not teach or suggest the use of hypoxia inducible TRE elements, as encompassed by the present claims.

Parr et al., 1997 is cited as disclosing adenoviral vectors for gene therapy which comprise a transgene operably linked to the E2F-1 promoter. Parr et al., does not teach or suggest replication competent adenovirus vectors comprising an HRE, as encompassed by the present claims.

One of skill in the art would not be motivated to combine gene therapy references such as Walther et al., the two Dachs et al. references, the Advani et al. reference and the Parr et al. reference, which describe vectors for targeted gene therapy, which by their nature are replication defective, with references such as Henderson (WO 97/01358) and Hallenbeck (WO 96/17053), which are cited as teaching conditionally replication competent adenovirus vectors. Taken together, Henderson (WO 97/01358) and Hallenbeck (WO 96/17053), do not suggest replication competent adenovirus vectors which comprise an HRE (i.e. a cell status-specific response element) and a cell type specific promoter for cell specific adenoviral gene expression, as encompassed by the present claims.

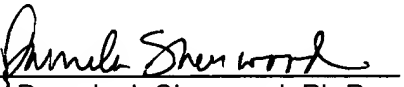
CONCLUSION

Applicants submit that all of the claims are now in condition for allowance, which action is requested. If the Examiner finds that a Telephone Conference would expedite the prosecution of this application, she is invited to telephone the undersigned at the number provided.

The Commissioner is hereby authorized to charge any other fees under 37 C.F.R. §§ 1.16 and 1.17 which may be required by this paper, or to credit any overpayment, to Deposit Account No. 50-0815, order number CELL-014.

Respectfully submitted,

Date: December 14, 2001

By: 
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APPENDIX

VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE SPECIFICATION

Page 7, lines 3-5, replace the paragraph with the following rewritten paragraph:

--In another embodiment, the cell status-specific TRE comprises a hypoxia responsive element.

Delete Figure 2.

Page 22, line 17 to page 23, line 4, replace the paragraph with the following rewritten paragraph:

--Another group of genes which are regulated by cell status are those whose expression is increased in response to hypoxic conditions. Bunn and Poyton (1996) *Physiol. Rev.* 76:839-885; Dachs and Stratford (1996) *Br. J. Cancer* 74:5126-5132; Guillemin and Krasnow (1997) *Cell* 89:9-12. Many tumors have insufficient blood supply, due in part to the fact that tumor cells typically grow faster than the endothelial cells that make up the blood vessels, resulting in areas of hypoxia in the tumor. Folkman (1989) *J. Natl. Cancer Inst.* 82:4-6; and Kallinowski (1996) *The Cancer J.* 9:37-40. An important mediator of hypoxic responses is the transcriptional complex HIF-1, or hypoxia inducible factor-1, which interacts with a hypoxia-responsive element (HRE) in the regulatory regions of several genes, including vascular endothelial growth factor, and several genes encoding glycolytic enzymes, including enolase-1. Murine HRE sequences have been identified and characterized. Firth et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:6496-6500. An HRE from a rat enolase-1 promoter is described in Jiang et al. (1997) *Cancer Res.* 57:5328-5335.--

Page 23, lines 5-9, replace the paragraph with the following rewritten paragraph:

--Accordingly, in one embodiment, an adenovirus vector comprises an adenovirus gene, preferably an adenoviral gene essential for replication, under transcriptional control of a cell status-specific TRE comprising an HRE.--

Page 26, lined 13-24, replace the paragraph with the following rewritten paragraph:

-- Example 1 provides a description of an adenovirus construct in which a composite TRE upstream of E1A consisting of an HRE and a prostate-specific TRE, PSA-TRE (which consists of enhancer sequences -5322 to -3738 fused to PSA promoter sequence -541 to +12; see U.S. Pat. Nos. 5,871,726; 5,648,478). Accordingly, in some embodiments, the invention provides an

adenovirus vector comprising an adenovirus gene essential for replication, preferably an early gene, preferably E1A or E1B, under transcriptional control of a TRE comprising an HRE and a prostate cell specific TRE, preferably comprising a PSA enhancer (preferably -5322 to -3738; or about 503 to about 2086 of SEQ ID NO:3 (bases about 503 to about 2086 of Figure 4), and a promoter, preferably comprising a PSA enhancer and a PSA promoter (about 5285 to about 5836 of SEQ ID NO:3).--

Page 35, lines 4-15, replace the paragraph with the following rewritten paragraph:

--The *PSE* and *PSA* TRE used in the present invention are derived from sequences depicted in Figure 4 (SEQ ID NO:3). The enhancer element is nucleotides about 503 to about 2086 of Figure 4 (SEQ ID NO:3). The promoter is nucleotides about 5285 to about 5836 of Figure 4 (SEQ ID NO:3). Accordingly, in some embodiments, the composite TRE comprises an HRE and a PSA-TRE comprises nucleotides about 503 to about 2086 of SEQ ID NO:3. In other embodiments, the composite TRE comprises an HRE [comprising SEQ ID NO:1] and a PSA-TRE comprises nucleotides about 503 to about 2086 of SEQ ID NO:3 and nucleotides about 5285 to about 5836 of SEQ ID NO:3. As described above, these composite (HRE/PSA) TREs may be operably linked to an adenovirus gene essential for replication, especially an early gene such as E1A or E1B. Example 1 describes such a construct.--

Page 49, lines 11-19, replace the paragraph with the following rewritten paragraph:

--An adenovirus vector containing a hypoxia response element (HRE) was generated. CN796, an adenovirus vector in which E1A is under the control of a composite TRE consisting of an HRE and a PSA-TRE, was made by co-transfecting CN515 with pBHG10. CN515 was constructed by inserting a 67 base pair fragment from HRE eno1 (Jiang et al. (1997) *Cancer Research* 57:5328-5335) [(SEQ ID NO:1; Figure 2)] into CN65 at the BglII site. CN65 is a plasmid containing an enhancer and promoter from the human PSA gene, consisting of an enhancer from -5322 to -3738 fused to a PSA promoter from -541 to +12. This is the PSA-TRE contained within plasmid CN706. Rodriguez et al. (1997) *Cancer Res.* 57:2559-2563.--

IN THE CLAIMS

1. **(amended)** A replication-competent adenovirus vector comprising an adenovirus gene essential for replication <u>selected from the group consisting of E1A, E1B and E4,</u> under transcriptional control of a <u>hypoxia responsive element (HRE)</u> [cell status-specific transcriptional regulatory element (TRE)], <u>wherein said HRE comprises a binding site for hypoxia inducible factor-1</u>.

Cancel claims 2-7.

8. **(amended)** The adenovirus vector of claim 1, wherein the [cell status-specific TRE] HRE is human.

Cancel claims 9-13.

14. **(amended)** The adenovirus vector of claim 1, [further comprising a cell type-specific TRE] wherein said adenovirus gene essential for replication is under the transcriptional control of a composite regulatory element comprising said HRE and a cell-type specific transcriptional regulatory element (TRE).

15. **(amended)** The adenovirus vector of claim 14, wherein [the] said cell type-specific TRE [is prostate cell specific] comprises a promoter.

16. **(amended)** The adenovirus vector of claim [15] 14, wherein [the prostate cell-specific TRE is a *PSA*-TRE] said cell type-specific TRE comprises an enhancer.

Cancel claims 17-20.

21. **(amended)** The adenovirus vector of claim [20] 14, wherein [the cell status-specific TRE comprises an HRE and the cell-type specific TRE is a PSA-TRE] said cell type-specific TRE comprises a prostate specific promoter and enhancer.

Cancel claims 22-23.

24. **(amended)** [The composition of claim 23,] A composition comprising:

a replication-competent adenovirus vector comprising an adenovirus gene essential for replication selected from the group consisting of E1A, E1B and E4, under transcriptional control of a hypoxia responsive element (HRE), wherein said HRE comprises a binding site for hypoxia inducible factor-1 [further comprising]; and

a pharmaceutically acceptable excipient.

get rid of

25. **(amended)** An isolated host cell comprising the adenovirus vector of claim 1.

26. **(amended)** A method of propagating adenovirus *in vitro* [specific for cells which allow a cell status-specific TRE to function, said method comprising combining an adenovirus according to claim 1 with the cells, whereby said adenovirus is propagated] the method comprising:

introducing into a cell an adenovirus vector comprising an adenovirus gene essential for replication selected from the group consisting of E1A, E1B and E4, under transcriptional control of a hypoxia responsive element (HRE), wherein said HRE comprises a binding site for hypoxia inducible factor-1 wherein said cell is maintained under hypoxic conditions *in vitro*, thereby expressing said adenovirus gene essential for replication;

wherein said adenovirus is propagated.

Cancel claims 27-31.

32. **(new)** The method of Claim 26, wherein said propagating of said adenovirus is cytotoxic to said cell.

33. **(new)** The method of Claim 32, wherein said cell is a tumor cell.

[CANCER RESEARCH 61, 3016–3021, April 1, 2001]

support for enablement

Gene Therapy Targeting for Hepatocellular Carcinoma: Selective and Enhanced Suicide Gene Expression Regulated by a Hypoxia-inducible Enhancer Linked to a Human α-Fetoprotein Promoter[1]

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ABSTRACT

We previously reported that the retroviral vector expressing the herpes simplex virus-thymidine kinase gene under the control of 0.3-kb human α-fetoprotein (AFP) gene promoter (AF0.3) provided the cytotoxicity to ganciclovir (GCV) in high-AFP-producing human hepatoma cells but not in low-AFP-producing cells. Therefore, specific enhancement of AFP promoter activity is likely to be required to induce enough cytotoxicity in low-AFP-producing hepatoma cells. In this study, we constructed a hybrid promoter, [HRE]AF, in which a 0.4-kb fragment of human vascular endothelial growth factor 5′-flanking sequences containing hypoxia-responsive element (HRE) was fused to AF0.3 promoter. By means of the reporter gene transfection assay, hypoxia-inducible transcriptions that were mediated by [HRE]AF promoter were detected in low- and non-AFP-producing human hepatoma cells, but not in nonhepatoma cells. When the herpes simplex virus-thymidine kinase gene controlled by [HRE]AF promoter was transduced into hepatoma and nonhepatoma cells by a retroviral vector, the exposure to 1% O_2 induced GCV cytotoxicity specifically in the hepatoma cells. Moreover, in nude mice bearing solid tumor xenografts, only the tumors consisting of the virus-infected hepatoma cells gradually disappeared by GCV administration. These results indicate that the hypoxia-inducible enhancer of the human vascular endothelial growth factor gene, which is directly linked to human AFP promoter, involves selective and enhanced tumoricidal activity in gene therapy for hepatocellular carcinoma.

INTRODUCTION

HCC[3] is one of the most common malignancies worldwide, especially in several areas of Asia and Africa (1). Because the development of HCC is strongly associated with chronic liver disease, particularly cirrhosis that occurs as a result of hepatitis B virus or HCV, the close follow-up of patients with chronic hepatitis B virus or HCV infection using imaging techniques and serum AFP assays has led to the detection of HCC at an early stage (2). However, even among patients in whom HCC is detected early, there are very few candidates for surgery because they generally lack a hepatic reserve as a result of the coexisting advanced cirrhosis (3). Moreover, clinical observations have shown that tumor recurrence rates are very high in patients with HCC who receive medical or surgical treatments. Thus, new treatment modalities must be pursued.

One new approach involves gene therapy using a retroviral vector that carries a kill or suicide gene such as the *HSV-tk* gene (4–6). Retroviruses can stably integrate their genes into proliferating cells, but they cannot integrate into quiescent cells. The killing effect of the HSV-tk gene product on the virus-infected cells is seen only in proliferating cells in which HSV-tk can efficiently phosphorylate nucleoside analogues and in which the phosphorylated products act as a chain terminator of DNA synthesis, thus leading to cell death (7–9). If the *HSV-tk* gene is driven by the tumor-specific promoter, it can cause selective ablation of tumor cells.

Many cancers often reexpress fetal or embryonic genes, and AFP gene expression is reactivated in HCC cells (10). There has been much progress in the characterization of *cis*- and *trans*-acting elements regulating human AFP gene expression (11–13). The hepatocyte-specific enhancers exist in a far upstream-regulatory region (−3.7- and −3.5-kb) of the AFP gene, and the position-dependent silencers are located between the enhancer regions and the hepatocyte-specific promoter region. To improve the selectivity of the antitumor effect in gene therapy for HCC, most investigators have used human AFP 5′-flanking sequences including enhancers or both enhancers and a silencer (4, 14–17). We previously reported on hepatoma-specific gene therapy using an AFP promoter alone to target AFP-producing hepatoma cells (5, 18, 19). The retroviral vector expressing the *HSV-tk* gene under the control of 0.3-kb human AFP gene promoter (AF0.3) provided cytotoxicity to GCV in high-AFP-producing human hepatoma cells (5). In contrast, the retroviral infection had little effect on the low-AFP-producing cells, because the 0.3-kb AFP promoter activity alone was not enough to express the HSV-tk gene in these cells. Because the level of AFP expression mostly depends on the activity of the enhancer, our AFP promoter/HSV-tk approach could decrease the cytotoxicity of GCV to stem cells, even if they express AFP, resulting in protection of the hepatic reserve. Recently, it has become possible to clinically detect HCCs at an early stage, and AFP expression in these tumors has been found to be relatively low (2). Furthermore, Northern blot analysis revealed only slight AFP expression in a noncancerous cirrhotic liver (20). Therefore, hepatoma-specific enhancement of AFP promoter activity is likely to be required to induce enough cytotoxicity in low-AFP-producing hepatoma cells.

Tumor development requires oxygen and nutrients, which are supplied through neovascularization. Angiogenic potential is, therefore, a prerequisite for tumor growth. Several growth factors (21–23), inflammatory cytokines (24, 25), and angiogenin (26) promote tumor angiogenesis, with the growth factors thought to be the most important of these. VEGF is vital for the neovascularization associated with tumor progression (27, 28). The expression of VEGF is induced by hypoxia through the HRE that exists in the VEGF 5′-flanking region (29). Recently, higher levels of VEGF expression have been found in HCC, which is generally considered to be a hypervascular tumor, than in corresponding nontumorous tissues (30–32).

In this study, we constructed a hybrid promoter ([HRE]AF) consisting of the hypoxia-inducible enhancer of the human VEGF gene. This hybrid promoter contained HRE directly linked to AF0.3 promoter. We evaluated the hepatoma-specific enhancement of GCV-

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[2] To whom requests for reprints should be addressed, at Department of Internal Medicine II, Miyazaki Medical College, 5200 Kihara, Kiyotake, Miyazaki, 889-1692, Japan. Phone: (81) 985-85-0987; Fax: (81) 985-85-5194.

[3] The abbreviations used are: HCC, hepatocellular carcinoma; AFP, α-fetoprotein; HSV, herpes simplex virus; tk, thymidine kinase; GCV, ganciclovir; luc, luciferase; NF-1, nuclear factor-1; HNF-1, hepatocyte nuclear factor-1; VEGF, vascular endothelial growth factor; HRE, hypoxia-responsive element; HIF-1, hypoxia inducible factor-1; HCV, hepatitis C virus.

mediated cytotoxicity that was induced by the *HSV-tk* gene under the control of [HRE]AF promoter *in vitro* and, more importantly, *in vivo*.

MATERIALS AND METHODS

Cell Culture. The amphotropic retrovirus packaging line PA317 was maintained in DMEM supplemented with 5% calf serum. The human hepatoma (PLC/PRF/5 and HLF) and cervical cancer (HeLa) cell lines were cultured in RPMI 1640 with 5% FBS, and the human fibrosarcoma (HT1080) was cultured in RPMI 1640 with 10% FBS.

All cell lines were incubated in a humidified 5% CO_2 and 95% air incubator at 37°C (normoxia). Hypoxia (1% O_2) was induced by placing cells in a 1% O_2-5% CO_2 and 94% N_2 incubator for 24 h at 37°C.

Luciferase Fusion Plasmids. The 1.8-kb fragment containing a 5′-flanking region and exon 1 of the human VEGF gene was cloned by PCR using 5′-ATTGCTGCATTCCCATTCTCA-3′ and 5′-GGGAATGGCAAGCAAA-AATAA-3′ as primers under the following conditions: denaturation at 94°C for 30 s; annealing at 55°C for 1 min; and extension at 72°C for 1 min (30 cycles). The 1.8-kb PCR products that corresponded to −1.6 kb to +0.2 kb of the human VEGF gene were cloned into pCR2.1 (Invitrogen, Carlsbad, CA) to make pCR-VEGFpro and then sequenced. The nucleotide sequence of the PCR product was identical with that of the human VEGF gene previously deposited with GenBank/EMBL Data Bank under accession no. M63971 (33).

To make luc fusion plasmids, pVE1.3-, pVE1.2- and pVE0.4-luc, the −1.3-, −1.2-, and −0.4-kb of the VEGF 5′-flanking region were released by *Bam*HI-, *Sac*I-, and *Bgl*II-*Hin*dIII double digestions, respectively, and inserted into the *Bgl*II- or *Sac*I-*Hin*dIII sites of pGL3-Basic, a firefly luc reporter vector (Promega, Madison, WI; Fig. 1*A*). To construct pVE0.8-luc, the −0.8-kb of the 5′-flanking region was released by *Pst*I-*Hin*dIII digestion, and the *Pst*I site of this fragment was blunt-ended. The resultant fragment was inserted into the *Sma*I-*Hin*dIII site of the pGL3-Basic vector.

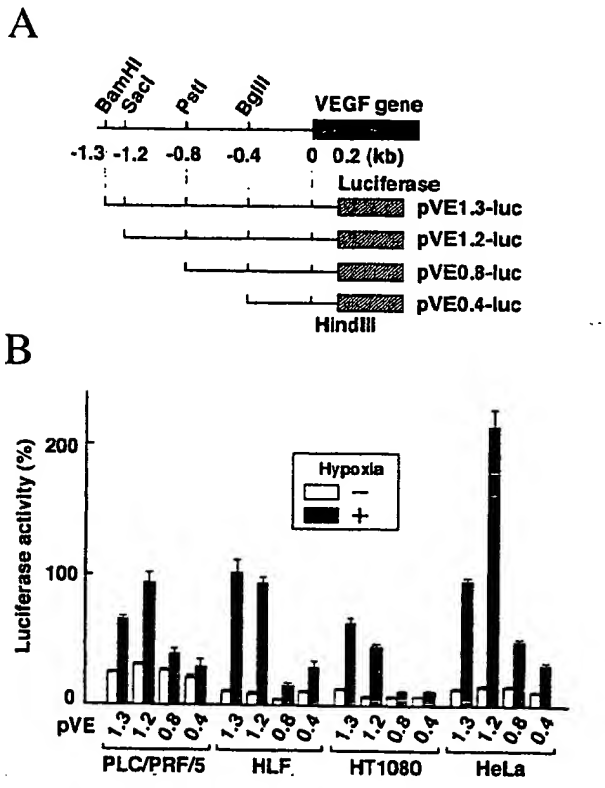

Fig. 1. Functional analysis of human VEGF 5′-flanking sequences in transient expression assays. *A*, luc fusion genes containing various lengths of human VEGF 5′-flanking sequences. Each deletion construct is designated by the restriction enzyme used to generate the 5′-end, the coordinate of which is indicated relative to the transcription initiation site. The 3′-end of each construct (+0.2 kb) was fused to the luc coding sequences. *B*, luc activities expressed in human hepatoma (PLC/PRF/5 and HLF) and nonhepatoma (HT1080 and HeLa) cells, which were transfected with the luc fusion genes shown in *A* and incubated under normoxic or hypoxic (1% O_2) conditions for 24 h. Transfection and analysis of luc activity were conducted as described in "Materials and Methods." Relative luc activity represents the mean firefly luc:*Renilla* luc ratio (n = 4); *bars*, SE.

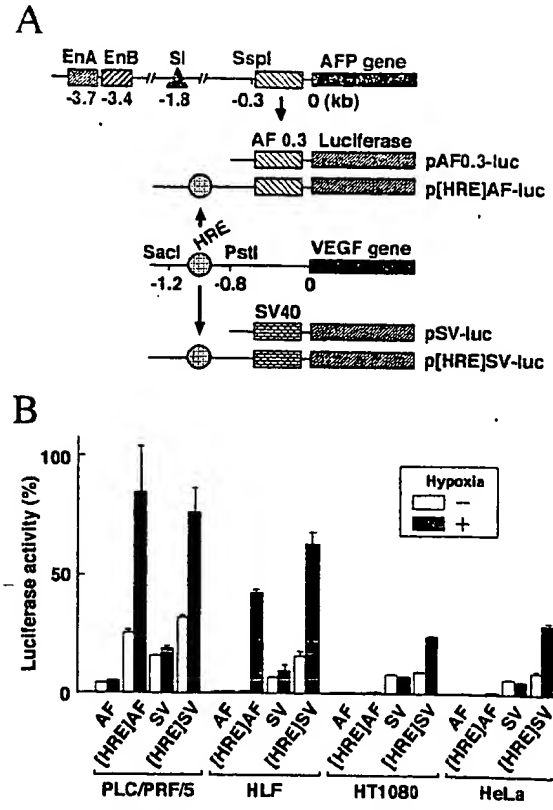

Fig. 2. Hepatoma-specific and hypoxia-inducible enhancement of luc expression mediated by the VEGF enhancer directly linked to the 0.3-kb human AFP promoter (AF0.3). *A*, luc reporter constructs in which luc expression is driven by AF0.3, SV40 promoters (SV40), and hybrid promoters consisting of the 0.4-kb fragment of human VEGF 5′-flanking sequences containing HRE fused to either AF0.3 or SV40 ([HRE]AF or [HRE]SV, respectively). *EnA*, *EnB*, and *Si*, enhancers A and B and a silencer of human AFP gene, respectively. *B*, luc expression in human hepatoma (PLC/PRF/5 and HLF) and nonhepatoma (HT1080 and HeLa) cells with or without 24 h exposure to hypoxia (1% O_2). Each column represents the mean relative luc activities (n = 4); *bars*, SE.

Human AFP gene 0.3-kb promoter was released from pBS-AF0.3 (5) by *Bam*HI-*Hin*dIII digestion, and inserted into the *Bgl*II-*Hin*dIII site of pGL3-Basic vector, resulting in pAF0.3-luc (Fig. 2*A*). The 0.4-kb *Sac*I-*Pst*I fragment between −1.2 and −0.8 kb relative to the cap site of human VEGF gene containing HRE was inserted into the *Sma*I site at the 5′-end of the AFP promoter of pAF0.3-luc and the SV40 promoter of the pGL-promoter vector (Promega), called pSV-luc in this study, resulting, respectively, in p[HRE]AF-luc and p[HRE]SV-luc (Fig. 2*A*).

Cell Transfection and Dual-Luciferase Reporter Assays. Cotransfection was performed using 1 μg of the luc fusion plasmids and 0.1 μg of pRL-TK, a *Renilla* luc control vector (Promega), per well in 24-well multiplates by lipofection methods (34). After transfection, the cells were incubated with the fresh medium under either the normoxic or hypoxic (1% O_2) condition. After 24 h of incubation, the cells were harvested and a Dual-Luciferase Reporter Assay was performed according to the instructions provided with the assay.

Retroviral Vectors. The retroviral vector, pLNAF0.3TK, which carries the *HSV-tk* gene under the control of human AFP 0.3-kb promoter, was produced as described previously (5). To create pLN[HRE]AFTK, the human AFP promoter of pLNAF0.3TK was replaced with [HRE]AF promoter, which was released from p[HRE]AF-luc (Fig. 3).

Production of Amphotropic Recombinant Retroviruses. Amphotropic retroviruses were produced as described previously (35). Briefly, the retroviral vectors pLNAF0.3TK and pLN[HRE]AFTK were transfected into PA317 amphotropic packaging cells using calcium phosphate precipitation. The cells were selected in the medium with 800 μg/ml G418 (Geneticin; Wako, Osaka, Japan), and G418-resistant colonies were cloned using cloning rings.

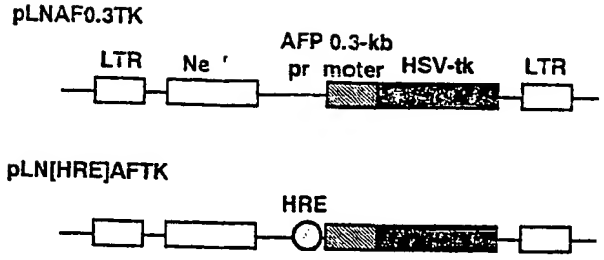

Fig. 3. Structure of the retroviral vectors, in which the *HSV-tk* gene is regulated by the AF0.3 promoter alone (pLNAF0.3TK) or by a hybrid gene consisting of the 0.4-kb VEGF hypoxia-inducible enhancer directly linked to AF0.3 (pLN[HRE]AFTK). *LTR*, long terminal repeat.

Retroviral Infection of Human Hepatoma and Nonhepatoma Cell Lines. Amphotropic recombinant retroviruses LNAF0.3TK and LN[HRE]-AFTK were harvested from the established producer cells. The hepatoma and nonhepatoma cells (1×10^6 cells) were incubated for 4 h in 3 ml of virus supernatants plus 4 ml of fresh medium containing 8 μg/ml Polybrene. The cells were then cultured in a complete medium containing G418 (400 μg/ml) for 2 weeks. G418-resistant pooled populations were subjected to further studies.

GCV-mediated Growth Inhibition Studies. GCV-mediated cell growth inhibition was determined by colorimetric quantitation of the viable cell number (36). The G418-selected pools of cells transduced with virus (5×10^4/well) were plated in 24-well multiplates. One day later (day 0), the cells were treated with 500 μl of fresh medium in the absence or presence of varying concentrations of GCV (Hoffman-La Roche, Basel, Switzerland). Each medium was replaced with corresponding fresh medium on days 2 and 4, and the cells were incubated under hypoxia (1% O_2) for 24 h every 2 days. To quantify the cell viability, the medium was replaced with 100 μl of fresh medium without GCV, then 10 μl of TetraColor ONE (SEIKAGAKU, Tokyo, Japan) were added to each well (day 6). After incubation for 1 h, the color reaction was quantitated using an automatic plate reader (Well-reader; Seikagaku) at 450 nm with a reference filter of 630 nm. The percentage of cell survival was calculated as

$$\text{Cell survival (\% control)} = (B/A) \times 100$$

where A is absorbance from the cells incubated with medium alone, and B is absorbance from the cells incubated with the medium containing various concentrations of GCV.

***In Vivo* Studies.** Four-week-old male BALB/c-*nu/nu* athymic mice were obtained from Charles River Japan (Yokohama, Japan). The mice were maintained under a constant room temperature (25°C) and were provided with free access to a standard diet and tap water throughout according to institutional guidelines. The study was approved by the ethical committee of Miyazaki Medical College (Miyazaki, Japan). The mice were inoculated s.c. in the right back with 1×10^6 cells of the LNAF0.3TK or N[HRE]AFTK virus-infected PLC/PRF/5 or HT1080 cell lines. One week later, when transplanted tumors had grown to ~5 mm in diameter, the mice were given i.p. injections of GCV (50 mg/kg) daily for 2 weeks. Serial changes in tumor volume were estimated twice a week after the start of the GCV treatment, on the basis of the Battelle Columbus Laboratory's protocol (Columbus, Ohio) (37) using the formula

$$VT\ (\text{mm}^3) = (L \times W^2) \times \tfrac{1}{2}$$

where VT is estimated tumor volume, and L and W are the length (mm) and width (mm) of the transplanted tumor, respectively.

RESULTS

Hypoxia-inducible Transcription Mediated by Human VEGF 5′-flanking Sequences in Hepatoma and Nonhepatoma Cells. To determine whether the 5′-flanking region of the VEGF gene mediates the transcriptional responses to cellular hypoxia, we constructed reporter plasmids in which VEGF 5′-flanking sequences were fused to luc coding sequences (Fig. 1*A*). This series of 5′ deletion mutants, pVE1.3-, pVE1.2-, pVE0.8-, and pVE0.4-luc, was transfected into human hepatoma (PLC/PRF/5 and HLF) and nonhepatoma cells (HT1080 and HeLa). After the cells were incubated in normoxia or hypoxia (1% O_2) for 24 h, a Dual-Luciferase Reporter Assay was performed.

The results are shown in Fig. 1*B*. pVE1.3- and pVE1.2-luc reporter plasmids mediated 2.5- to 16-fold greater levels of luc expression in both hepatoma and nonhepatoma cells that were exposed to hypoxia (1% O_2) than did cells that were exposed to normoxia. In contrast, pVE0.8- and pVE0.4-luc induced 60 to 80% less transcription than pVE1.3- and pVE1.2-luc in hypoxic cells, although, in comparison to cells exposed to normoxia, the luc expression mediated by pVE0.8- and pVE0.4-luc was weakly increased by hypoxia. These results demonstrate that VEGF 5′-flanking sequences mediate the transcriptional response to hypoxia in both hepatoma and nonhepatoma cells and that the region between 1.2 and 0.8 kb 5′ to the VEGF transcription initiation site is specifically involved in mediating hypoxia-inducible transcription, acting as the hypoxia-inducible enhancer. These results also agree with a previous report that described HRE as existing −1.0 kb upstream of the human VEGF gene (29).

Hepatoma-specific and Enhanced Transcriptional Activity of [HRE]AF Promoter. To obtain hypoxia-inducible and hepatoma-specific transcription, we prepared a hybrid promoter, [HRE]AF, in which the 0.4-kb fragment between 1.2 and 0.8 kb 5′ to the VEGF transcription initiation site was directly linked to the human AFP 0.3-kb promoter, AF0.3 (Fig. 2*A*). By transient transfection assay, transcription induced by AF0.3 or [HRE]AF was analyzed in human hepatoma or nonhepatoma cells under either normoxic or hypoxic conditions.

As shown in Fig. 2*B*, in PLC/PRF/5 cells (low-AFP-producing hepatoma cells) exposed to 1% O_2, the luc expression mediated by [HRE]AF promoter 22-fold greater than that mediated by AF0.3 promoter, although the addition of the VEGF hypoxia-inducible enhancer to AF0.3 also resulted in a 4-fold increase in luc expression in the normoxic cells. In HLF cells (non-AFP-producing hepatoma cells) without 1% O_2 exposure, both AF0.3 and [HRE]AF promoter slightly induced luc expression. However, in hypoxic HLF cells, [HRE]AF promoter mediated an increase in luc expression that was 100-fold greater than that mediated by AF0.3 promoter. The levels of luc expression induced by the [HRE]AF promoter were similar to those induced by the [HRE]SV promoter in these hypoxic hepatoma cells. In nonhepatoma cells, HT1080 and HeLa, luc expression driven by SV and [HRE]SV promoters was detected, and the expression of [HRE]SV was enhanced by 1% O_2 exposure, whereas transcription mediated by AF0.3 and [HRE]AF was not detected in the normoxic or hypoxic cells. These results indicate that hypoxia-inducible transcription through the VEGF enhancer directed by AF0.3 promoter is circumscribed within hepatoma cells, even if they do not express AFP, although the VEGF enhancer is functional in both hepatoma and nonhepatoma cells.

Specific and Enhanced Cytotoxicity of GCV in LN[HRE]AFTK Virus-infected Hepatoma Cells by Hypoxia. We constructed hybrid genes consisting of *HSV-tk* genes under the control of either AF0.3 or [HRE]AF promoter and inserted them into retroviral vectors, thus producing LNAF0.3TK and LN[HRE]AFTK, respectively (Fig. 3). The low- and non-AFP-producing hepatoma cells (PLC/PRF/5 and HLF, respectively) and nonhepatoma cells (HT1080 and HeLa) were infected with these recombinant retroviruses. Because the transduction efficiency might contribute to the results, G418-resistant pooled populations were used for *in vitro* and *in vivo* studies of GCV-mediated cytotoxicity to precisely evaluate the activity and specificity of the promoters.

LNAF0.3TK and LN[HRE]AFTK virus-infected cells were exposed to both 1% O_2 for 24 h every 2 days and to varying concen-

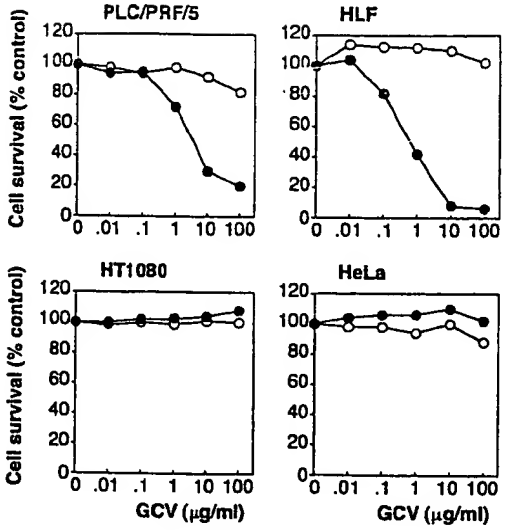

Fig. 4. *In vitro* cytotoxic effect of GCV in the hypoxic cells infected with either LNAF0.3TK or LN[HRE]AFTK retrovirus. Cells infected with the recombinant retroviruses were incubated with various concentrations of GCV for 6 days and exposed to 1% O_2 for 24 h every 2 days, followed by cell survival quantitation as described in "Materials and Methods." Data are representative of at least two separate experiments; each point represents the mean (n = 4) and is expressed as a percentage relative to hypoxic untreated cells. ○, LNAF0.3TK-infected cells; ●, LN[HRE]AFTK-infected cells.

trations of GCV for 6 days. Their survival curves are shown in Fig. 4. LNAF0.3TK infection induced weak GCV cytotoxicity only in low-AFP-producing hepatoma cells (PLC/PRF/5), and induced no cytotoxicity in non-AFP-producing hepatoma (HLF) and nonhepatoma cells (HT1080 and HeLa). In contrast, LN[HRE]AFTK infection sensitized both low- and non-AFP-producing hepatoma cells to GCV but did not sensitize nonhepatoma cells.

These results demonstrate that the HSV-tk/GCV system under the control of [HRE]AF promoter is able to induce enhanced hepatoma-specific cytotoxicity in hypoxic cells.

Hepatoma-specific Induction of HSV-tk/GCV System in Tumor-bearing Athymic Mice. Tumor cells are exposed to hypoxia in solid tumors. Therefore, we analyzed LNAF0.3TK or LN[HRE]AFTK retrovirus-mediated cytotoxicity to GCV in athymic mice bearing solid tumor xenografts. Because the PLC/PRF/5 and HT1080 cells were transplantable in athymic mice, the LNAF0.3TK or LN[HRE]AFTK virus-infected tumor cells were inoculated s.c. in the mice. At 1 week after s.c. inoculation, the mice were given GCV for 14 days. The LNAF0.3TK virus-infected PLC/PRF/5 cells grew rapidly, regardless of GCV administration, reaching tumor volumes that were ~5–6-fold greater during the observation period than during pretreatment (Fig. 5). The tumors consisting of LN[HRE]AFTK virus-infected PLC/PRF/5 cells gradually disappeared as a result of the 14-day GCV treatment, but without GCV administration they grew as rapidly as the LNAF0.3TK virus-infected PLC/PRF/5 cells (Fig. 5). On the other hand, the growth of s.c. tumors consisting of LNAF0.3TK or LN[HRE]AFTK virus-infected HT1080 cells was not inhibited by the 14-day GCV administration.

These results indicate that the [HRE]AF/HSV-tk approach induces specific enhancement of the cytotoxicity to GCV in solid tumors made up of low-AFP-producing hepatoma cells.

DISCUSSION

In the present study, we transduced the HSV-tk gene under the control of the human AFP promoter (AF0.3) or a hybrid promoter ([HRE]AF) into low- and non-AFP-producing hepatoma cells and into nonhepatoma cells using the LNAF0.3TK and LN[HRE]AFTK retroviruses, respectively. The cytotoxicity to GCV that was induced by LNAF0.3TK infection was, *in vitro*, detected weakly in low-AFP-producing hepatoma cells, and not at all in non-AFP-producing hepatoma and nonhepatoma cells. In addition, the growth of tumor xenografts of LNAF0.3TK virus-infected low-AFP-producing hepatoma cells in nude mice was not affected by systemic GCV treatment for 14 days. On the other hand, the LN[HRE]AFTK infection induced GCV cytotoxicity in low- and non-AFP-producing hepatoma cells exposed to 1% O_2, and, when mice bearing solid tumors consisting of LN[HRE]AFTK-infected low-AFP-producing hepatoma cells were treated by GCV administration, a complete regression of the tumors was observed without any signs of overt toxicity. Importantly, the LN[HRE]AFTK infection did not sensitize nonhepatoma cells to GCV either *in vitro* or *in vivo*, although the VEGF hypoxia-inducible enhancer was functional in these cells that had been exposed to 1% O_2. The 0.3-kb AFP promoter contains a glucocorticoid response element and two binding sites for HNF-1, a hepatocyte-specific transcriptional factor, and a TATA box (5, 13, 38) but does not have a typical CCAAT sequence (39). Recent studies reported that one of the HNF-1 binding sites (−132 bp to −116 bp) was also recognized by NF-1 and CCAAT/enhancer binding proteins that function actively during postnatal liver growth (40, 41). Although NF-1 competes with HNF-1 in the HNF-1 binding site in non-AFP-producing hepatoma cells as well as in adult hepatocytes, the AFP promoter shows a trace of activity in non-AFP-producing hepatoma cells (42). Therefore, the VEGF hypoxia-inducible enhancer can selectively activate the AF0.3 promoter under the hypoxic condition in HLF non-AFP-producing hepatoma cells but not in HT1080 and HeLa nonhepatoma cells.

Because HCC frequently occurs in patients with liver cirrhosis and the prognosis of patients is influenced by the hepatic reserve, the selectivity of antitumor effects is vital for gene therapy for HCC. To improve selectivity, gene therapy based on targeted gene transfer or the specific expression of transfected genes to hepatoma cells is currently being investigated. However, most investigators have used human AFP 5′-flanking region including the enhancers and silencer (4, 14–17). We previously reported on hepatoma-specific gene therapy using the 0.3-kb AFP promoter alone to target AFP-producing hepatoma cells (5, 18, 19). Also, we demonstrated that a human AFP

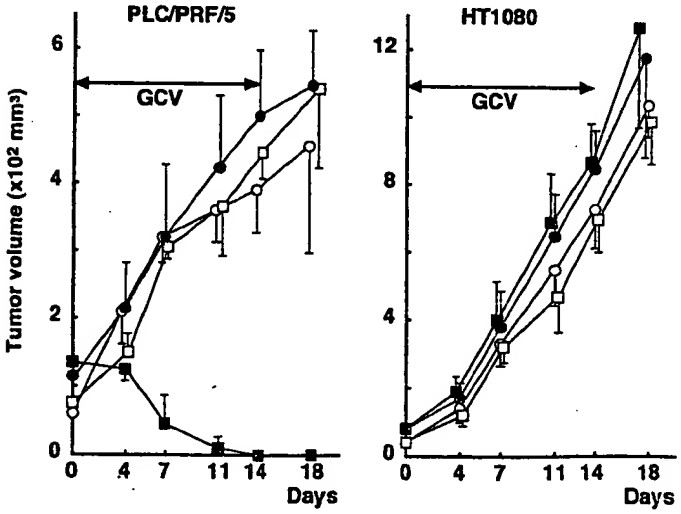

Fig. 5. *In vivo* cytotoxic effect of GCV in solid tumor xenografts consisting of human hepatoma (PLC/PRF/5) and nonhepatoma (HT1080) cells infected with either LNAF0.3TK or LN[HRE]AFTK in nude mice. The mice bearing the s.c. tumors formed out of virus-infected cells were treated by daily i.p. injection of 50 mg/kg GCV for 2 weeks. Tumor size was measured twice a week. Each point is the mean (n = 3-5); *bars*, SE. ▲ and ○, tumors of LNAF0.3TK-infected cells with and without GCV, respectively; ■ and □, tumors of LN[HRE]AFTK-infected cells with and without GCV, respectively.

enhancer directly linked to its promoter and a variant type of AFP promoter with G-to-A substitution at nucleotide −119 was able to sensitize low-AFP-producing hepatoma cells to GCV (43, 44). However, the addition or modification of *trans*- and *cis*-acting elements from AFP 5′-flanking sequences, to achieve sufficient cytotoxicity in low-AFP-producing hepatoma cells, must also induce a killing effect on hepatic stem cells that also express AFP and appear in the injured liver, thus resulting in damage of the hepatic reserve and a poor prognosis for patients with HCC.

Aggressive tumors often have an insufficient blood supply, partly because tumor cells grow faster than the endothelial cells that make up the blood vessels and partly because the newly formed vascular supply is disorganized. This situation produces areas with reduced oxygen tension and nutrient deprivation. Hypoxia is a powerful modulator of gene expression. An important mediator of these responses is the interaction of a transcriptional complex termed HIF-1 with its cognate DNA recognition site, HRE. Molecular analysis of HIF-1 revealed that the DNA-binding complex consists of a heterodimer of two basic helix-loop-helix proteins, HIF-1α and HIF-1β (45). HIF-1α was a newly described protein, but HIF-1β had already been recognized as the dimerization partner of the aryl hydrocarbon receptor in the xenobiotic response, where it was termed the aryl hydrocarbon receptor nuclear translocator (46). Recently, several investigators have shown the potential of exploiting tumor-specific conditions for the targeted expression of therapeutic genes in cancer therapy (47–51). Dachs *et al.* (47) fused three copies of HRE from the mouse phosphoglycerate kinase-1 gene to the 9-27 promoter to control the expression of the bacterial cytosine deaminase or the marker, CD2, gene. When HT1080 cells were transfected with the cytosine deaminase or CD2 gene under the control of the phosphoglycerate kinase-1 HRE, these cells were found to be more sensitive to the prodrug 5-fluorocytosine than were the parental cells after exposure to hypoxia, and the CD2 expression was up-regulated in a solid tumor xenograft in nude mice. In another study, Gazit el al. (48) reported on starvation-inducible suicide gene therapy under the control of glucose-regulated protein 78 promoter, using a murine fibrosarcoma model. The strategies described in these reports are designed to use tumor-specific conditions, such as hypoxia or starvation, that exist in almost all solid tumors regardless of their origin or location, to control the expression of heterologous genes. In the approach described in the present study, when the VEGF hypoxia-inducible enhancer was directly linked to the AF0.3 promoter, specific enhancement of transfected gene expression was induced in hypoxic hepatoma cells *in vitro* and in solid tumor xenografts consisting of low-AFP-producing hepatoma cells. However, hypoxia-inducible enhancement of AFP promoter activity mediated by the VEGF enhancer was not detected in nonhepatoma cells *in vitro* or *in vivo*. These results demonstrate that hypoxia-inducible enhancement of transgene expression through VEGF enhancer could be directed to be hepatoma specific by AFP promoter.

We used the 0.4-kb fragment containing HRE between −1.2 and −0.8 kb relative to the cap site of the human VEGF gene for hepatoma-specific enhancement of AFP promoter activity. A 47-bp sequence located 985–939 bp 5′ to the VEGF transcription initiation site mediates hypoxia-inducible expression that is directed by SV40 promoter (29). We constructed several hybrid promoters in which various deletions of the 0.4-kb fragment containing VEGF HRE or three copies of 24-bp VEGF HRE core sequence were directly linked to the 0.3-kb AFP promoter and examined those transcriptional activities. The deletions and the three copies of the HRE core sequence mediated only 30–70% less reporter gene expression than did the [HRE]AF promoter in hypoxic hepatoma cells (data not shown), probably because the HRE core sequence and the *trans*- and *cis*-acting elements existing in the AFP promoter were too close and because the transcription factors, such as HIF-1, HNF-1, and NF-1, might be unable to bind to these regions cooperatively.

Hypoxia-inducible expression of VEGF plays a central role in neovascularization, which is essential for tumor growth beyond 1–2 mm^3. Several reports have shown that VEGF transcripts are overexpressed in HCC tissues as compared with noncancerous liver tissues, which express VEGF less intensely (30–32). Recently, other investigators have demonstrated by semiquantitative RT-PCR that there was no difference in VEGF expression between HCC and liver tissues with chronic HCV infection and that cirrhotic livers had significantly higher VEGF expression than did noncirrhotic livers (52, 53). Therefore, when the transfected genes are controlled by VEGF 5′-flanking full sequences, the expression could be induced in both HCC and noncancerous tissues. However, in the noncancerous tissues in which VEGF expression is detected, inflammatory cell infiltration is apparent, and the VEGF expression is stimulated not only by hypoxia but also by various cytokines and growth factors (32). Furthermore, Maxwell *et al.* (54) have described that HIF-1 or a closely related heterodimer involving HIF-1β/aryl hydrocarbon receptor nuclear translocator is the major mediator of VEGF expression within solid tumors, despite the multiple regulatory mechanisms operating on the VEGF gene. Therefore, the use of the VEGF hypoxia-inducible enhancer could allow us to exploit tumor hypoxia to obtain selective expression of genes and to achieve tumor-specific rather than tissue-specific targeting. Also, the [HRE]AF hybrid promoter could be useful for inducing targeted expression of therapeutic genes in HCCs that produce various amounts of AFP and for preventing their expression in hepatic stem cells that also express AFP.

The activity of the AFP promoter is originally weak, but we found that adding the VEGF hypoxia-inducible enhancer directly to the AFP promoter resulted in sufficient and specific expression of transfected genes, even in the solid tumors consisting of low-AFP-producing cells. In addition, transcriptional activation via the VEGF hypoxia-inducible enhancer, which contains HRE, is mediated by HIF-1, the activation of which occurs in the hypoxic cells of solid tumors. Thus, our construct can mediate efficient therapeutic gene expression selectively in human hepatoma cells, although *in vivo* studies using G418-unselected cells would be more relevant, and the effect of our construct on liver stem cells needed to be examined experimentally if the clinical application was being considered. This selective mediation would shed some light on strategies for gene therapy that targets HCC.

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A Hypoxia-Regulated Adeno-Associated Virus Vector for Cancer-Specific Gene Therapy[1]

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Abstract

The presence of hypoxic cells in human brain tumors is an important factor leading to resistance to radiation therapy. However, this physiological difference between normal tissues and tumors also provides the potential for designing cancer-specific gene therapy. We compared the increase of gene expression under anoxia (<0.01% oxygen) produced by 3, 6, and 9 copies of hypoxia-responsive elements (HRE) from the erythropoietin gene (*Epo*), which are activated through the transcriptional complex hypoxia-inducible factor 1 (HIF-1). Under anoxic conditions, nine copies of HRE (9XHRE) yielded 27- to 37-fold of increased gene expression in U-251 MG and U-87 MG human brain tumor cell lines. Under the less hypoxic conditions of 0.3% and 1% oxygen, gene activation by 9XHRE increased expression 11- to 18-fold in these cell lines. To generate a recombinant adeno-associated virus (rAAV) in which the transgene can be regulated by hypoxia, we inserted the DNA fragment containing 9XHRE and the *LacZ* reporter gene into an AAV vector. Under anoxic conditions, this vector produced 79- to 110-fold increase in gene expression. We believe this hypoxia-regulated rAAV vector will provide a useful delivery vehicle for cancer-specific gene therapy. *Neoplasia* (2001) 3, 255–263.

Keywords: hypoxia, brain tumor cells, hypoxia-responsive element, adeno-associated virus, gene therapy.

Introduction

Solid tumors are heterogeneous and are composed of physiologically distinct subpopulations of cells. One important feature of human solid tumors is the presence of a hypoxic microenvironment [1]. Pioneering studies by Thomlinson and Gray [2] demonstrated that hypoxic cells arose in tumors whenever tumor growth produces cells that are >150 μm from a blood vessel. Clinical studies using an oxygen electrode to measure the oxygen levels in patients' brain tumor tissues have found a significant number of hypoxic cells in every tumor examined [3]. Hypoxia in solid tumors confers resistance to standard radiotherapy and chemotherapy [4,5]. Furthermore, oxygen-deprived tumor cells are predisposed to a more malignant phenotype, as characterized by increases in local invasion, metastatic spread, and genetic instability [6,7]. Inhibition of cellular proliferation by hypoxia also may contribute to a resistant phenotype, because current cancer treatment modalities are primarily effective against rapidly dividing cells. In brief, hypoxia within tumors most likely contributes to a poor therapeutic outcome, regardless of which cancer treatment is used [7].

In addition to conferring resistance to cancer therapy, recent clinical and experimental studies have also suggested that the cellular response to hypoxia can result in dramatic alterations in the expression of a variety of genes, many of which may lead to more aggressive phenotypes. For example, hypoxia in solid tumors mediates selection of cells with diminished apoptotic potential and with genetic alterations such as loss of the p53 tumor suppressor gene or overexpression of the apoptotic inhibitor protein Bcl-2 [8].

Studies of molecular responses to hypoxia have identified the major mediator for cellular hypoxic responses as a transcriptional activator hypoxia-inducible factor 1 (HIF-1) [9]. HIF-1 is a heterodimeric basic helix–loop–helix (bHLH) protein consisting of two subunits, HIF-1α and HIF-1β. HIF-1α is the unique, O_2-regulated subunit that determines HIF-1 activity [10]. Under hypoxic conditions, HIF-1 binds to the hypoxia-responsive element (HRE) in the enhancer region of its target genes and turns on gene transcription. HIF-1 responsive genes include glycolytic and gluconeogenic enzymes in energy metabolism, vascular endothelial growth factor (VEGF), transcription factors, glucose transporters, tyrosine hydroxylase and erythropoietin (Epo), a hormone that regulates erythropoiesis in accordance with the oxygen-carrying capacity of the blood. The core consensus sequence for HRE has been identified as (A/G)CGT(G/C) [11].

Several studies have demonstrated overexpression of HIF-1α in cancer cells and tissues. For example, increased

Abbreviations: HRE, hypoxia-responsive element; HIF-1, hypoxia-inducible factor 1; rAAV, recombinant adeno-associated virus; bHLH, basic helix–loop–helix; Epo, erythropoietin; CMEM, complete growth medium; MEM, Eagle's minimum essential medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; SDS, sodium dodecylsulfate; β-gal, β-galactosidase; MOI, multiplicity of infection; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactosidase; VEGF, vascular endothelial growth factor

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expression of HIF-1α protein has been detected in rat and human prostate cancer cell lines [12]. Recently, immunohistochemical analysis of HIF-1α has revealed increased expression in a variety of tumor types compared with the respective normal tissues, including brain, colon, breast, gastric, lung, skin, ovarian, pancreatic, prostate, and kidney [13,14]. These results suggest that HIF-1α overexpression is associated with tumor progression and invasion, possibly by increased cell growth and metastatic potential.

Although the presence of hypoxic cells in human brain tumors is an important factor leading to resistance to cancer treatments, this physiological difference between normal tissues and tumors also provides the potential for designing cancer-specific gene therapy protocols [4]. Over the last 10 years a number of viral vectors including adeno-associated virus (AAV) have been developed to deliver transgenes that are potentially useful for treating central nervous system malignancies [15]. However, a fundamental problem for cancer gene therapy is the lack of tumor specificity; proteins that are therapeutic in malignant cells also may be harmful to surrounding normal tissue. One way to circumvent this problem is to use transcriptionally targeted vectors that can restrict the expression of the therapeutic proteins primarily to cancer cells [16].

We have previously demonstrated the feasibility of this approach by employing a trimer of a minimal (31 bp) HRE from the human Epo gene to regulate the expression of the pro-apoptotic BAX gene [17]. In the present study, we sought to improve the ratio of hypoxic induction of gene expression. We compared the induction of gene expression from constructs containing an SV40 minimal promoter and 3X, 6X, or 9XHRE. Our results indicate that 6XHRE produced a similar increase in gene expression under anoxic conditions as observed earlier for 3XHRE [17]. However, 9XHRE increased the amount of gene expression markedly in both cell lines, as compared with the lower HRE copy numbers. We subsequently characterized induction of gene expression by 9XHRE under intermediate oxygen concentrations. In stable clones transfected with a construct containing 9XHRE and the suicide gene BAX, gene expression was increased under hypoxic conditions. Finally, we report development of a novel recombinant adeno-associated virus (rAAV) in which the expression of a transgene is under the control of HREs and occurs only under hypoxic conditions.

Materials and Methods

Plasmid Construction

Based on the published HRE sequence from the 3′ enhancer region of the Epo gene [18], we designed pairs of oligonucleotides that contain three tandem repeats of the HRE (G CCC *TAC GTG CTG* TCT CAC ACA GCG CCT GTC) [17]. In addition, 6X and 9XHREs were generated by tandem ligation of the 3XHRE oligonucleotide pairs. These oligonucleotides were inserted into the multiple cloning sites of the mammalian expression vector pβgal-promoter (Clontech, Palo Alto, CA), which contains an enhancerless SV40 promoter situated upstream of the *LacZ* gene. The resulting constructs were named pH3LacZ, pH6LacZ, and pH9LacZ, respectively (Figure 1). To construct pH9BAX, the *LacZ* gene in pH9LacZ was deleted using *Hin*dIII and *Nde*I enzymes and replaced with a murine pro-apoptotic BAX cDNA fragment flanked by *Hin*dIII and *Nde*I sites. To construct the plasmid pH9LacZ-AAV, the DNA fragment containing 9XHRE and the *LacZ* gene flanked by restriction enzyme sites *Sma*I and *Sal*I were inserted into pSSV9 (Figure 1). The plasmid pCMVLacZ-AAV was similarly

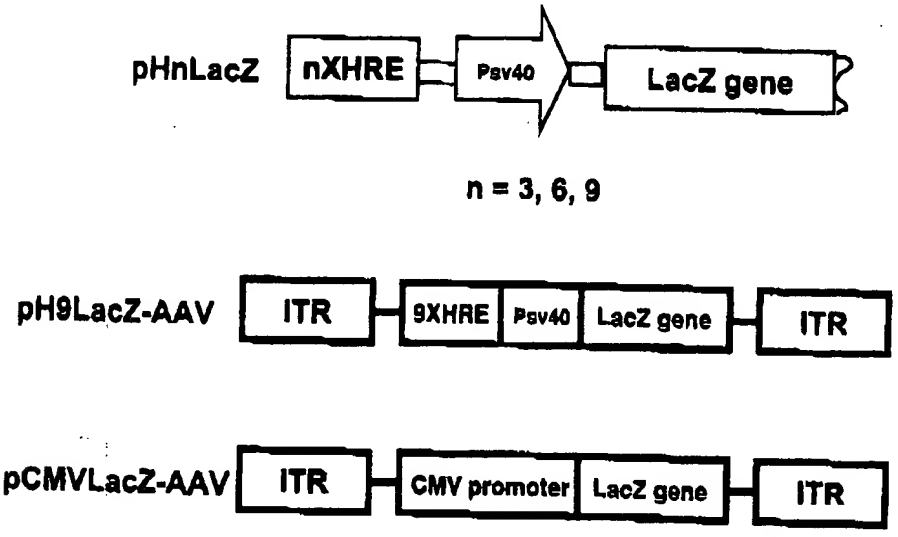

Figure 1. *Structural map of plasmids pH3LacZ, pH6LacZ, pH9LacZ, and rAAV vectors. In pHnLacZ plasmids, the reporter gene LacZ was under the transcriptional control of different copy numbers of HRE and an SV40 minimal promoter. In pH9LacZ-AAV, the LacZ with nine copies of HRE and the SV40 minimal promoter was inserted between the two inverted repeat regions (ITR). The control vector pCMVLacZ-AAV contains a CMV promoter upstream of the LacZ gene.*

constructed by inserting the CMV promoter and *LacZ* gene fragment into pSSV9 (Figure 1) [19].

Cell Culture

Human glioblastoma cell lines U-251 MG and U-87 MG and human embryonic kidney cell line 293 were maintained in complete growth medium (CMEM), which consists of Eagle's minimum essential medium (MEM) supplemented with 1% nonessential amino acids and 10% fetal bovine serum (FBS). Cultures were incubated in an humidified atmosphere containing 5% CO_2 at 37°C.

Induction of Hypoxia in Tumor Cells

Cells were seeded into glass petri dishes, allowed to attach overnight and then placed into gastight aluminum gassing jigs. The jigs were subjected to five rounds of evacuation and flushing with either 95% air and 5% CO_2 (oxic conditions) or 95% N_2 and 5% CO_2 (anoxic conditions) on a shaking platform at room temperature. We produced other hypoxic mixtures by using known mixtures of 95% O_2/5% CO_2 and 95% N_2/5% CO_2, which were ordered from the manufacturer (Nellcor Puritan Bennett, Pleasanton, CA). After the last round of gas exchange, the jigs were placed in a 37°C incubator and incubated for 16 hours.

Transient Transfection

Cells (4×10^6) were plated into 6-cm glass petri dishes and grown for 16 to 18 hours until cell growth reached log phase and cell density was ~50% confluent. Multiple sets of dishes in either duplicate or triplicate were prepared for each plasmid sample. Transfections were performed using 6 μl of lipofectamine (2 mg/ml, Gibco BRL Life Technologies, Gaithersburg, MD), 5 μg of plasmid containing *LacZ* and various copy number of HREs, and 0.5 μg of DNA containing the luciferase reporter gene (to correct for transfection efficiency) in 2 ml of serum-free CMEM for 5.5 hours. Then, the transfection solution in each dish was replaced with 4 ml of fresh CMEM and incubated at 37°C for 24 hours. One set of dishes was grown under normal oxic conditions and the other sets were subjected to different hypoxic conditions for 16 hours. Cells were then harvested and assayed for β-galactosidase (β-gal) activity by the enzymatic method described below.

Stable Transfection

Cells were stably transfected with pH9BAX using lipofectamine reagent. For U-251 MG, 2×10^5 cells/well were transfected with 5 μg of DNA and 7.5 μl of lipofectamine. Thirty-six hours after addition of the DNA and lipofectamine, growth medium was changed and cells were incubated in growth medium containing 600 μg/ml G418 (Gibco BRL Life Technologies) to select for stably transfected clones. Growth medium was replaced with fresh medium containing G418 every 2 to 3 days to maintain the concentration of active G418. After 10 to 12 days of G418 selection, individual surviving clones were isolated and expanded in six-well dishes. Stably transfected clones were maintained in medium containing 600 μg/ml G418.

rAAV Production

rAAV was produced by using the three-plasmid cotransfection system as described previously [20]. Briefly, human embryonic kidney 293 cells were grown in MEM containing 10% FBS in 15-cm plastic petri dishes to 70% confluence. To generate AAVH9LacZ virus, the cells were cotransfected with 17 μg of plasmid pH9LacZ-AAV per dish along with 17 μg of plasmid pHLP19 and 17 μg of plasmid pLadeno5 per dish. pHLP19 has AAV *rep* and *cap* genes, which provide the *trans* functions of rAAV. pLadeno5 has the adenoviral VA, E2A and E4 regions that mediate rAAV replication. To generate AAVCMVLacZ virus, plasmid pCMVLacZ-AAV was used in place of pH9LacZ-AAV in the above cotransfection process. The medium was changed after 16 hours to CMEM. After an additional 24 hours, the cells were collected and lysed by three freeze–thaw cycles. Viral supernatants were generated by centrifugation at 10,000×*g* for 5 minutes and further purified by CsCl-gradient ultracentrifugation; the titer for each rAAV was determined by dot blot assay. This assay provides a titer of total number of particles per unit volume. The supernatant containing rAAV was stored in aliquots at −80°C and thawed for use immediately before each experiment.

rAAV Infection

U-251 MG cells were grown to 70% confluence in 6-cm glass petri dishes. For infection, the growth medium was replaced with rAAV-containing medium and dishes were incubated for 48 hours at 37°C in 5% CO_2. At the end of the incubation period, cells were subjected to β-gal expression analysis using either an enzymatic assay or an X-gal *in situ* staining assay.

Western Immunoblotting

Total cellular protein was extracted from cell lines by lysing cells in 0.5 ml of phosphate-buffered saline (PBS) containing 0.5% deoxycholate, 1% Nonidet P-40 and 0.1% sodium dodecylsulfate (SDS) at pH 7.4. To reduce sample viscosity, DNA was sheared by passage through an 18-gauge needle. Samples were boiled for 3 minutes, immediately cooled on ice, and spun for 5 minutes at 10,000 rpm in a microcentrifuge to remove insoluble material. Supernatants were assayed for protein concentration using the Bradford method [21] (Bio-Rad, Hercules, CA) and then stored at −70°C. Before electrophoresis, sample buffer containing bromophenol blue was added to reach a final concentration of 10% glycerol, 2% SDS, and 50 mM Tris–Cl, pH 6.8. Protein samples were boiled for 3 minutes and cooled before loading. Equal amounts of total protein were separated on an SDS–polyacrylamide gel for 1.5 hours at 150 V using a Bio-Rad minigel apparatus (Bio-Rad). The protein gel was transferred to a polyvinylidene difluoride membrane (Amersham, Buckinghamshire, UK) for 1 hour at 100 V in transfer buffer containing 25 mM Tris–HCl, 40 mM glycine, and 20% methanol at pH 8.3. The membrane was stained for total protein using Ponceau-S (Sigma, St. Louis, MO). M*m*-brane was blocked with PBS containing 5% skim milk powder and 0.05% Tween 20 at pH 7.4. After incubation for 1

hour at room temperature, fresh blocking solution containing either the anti-human HIF-1α antibody (Novus Biologicals, Littleton, CO) or the anti-murine BAX antibody (Genzyme, Cambridge, MA) was added and the membrane was incubated for 1 hour. The membrane was washed three times in PBS containing 0.05% Tween 20 at room temperature and followed by incubation with a peroxidase-labeled secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 hour at room temperature. After three washes in PBS plus 0.05% Tween 20, the membrane was incubated for 1 minute in enhanced chemiluminescence buffer (Amersham) and proteins were visualized on an X-ray film using exposure times varying from 30 seconds to 5 minutes.

β-gal and Luciferase Assays

Transfected cells were washed with PBS, removed from the glass surface using a rubber policeman and transferred into Eppendorf tubes. Cells were lysed using the "freeze–thaw" method, centrifuged, and the supernatants were collected. The supernatants were assayed for β-gal activity using a chemiluminescent method according to the manufacturer's directions (Clontech). Briefly, an aliquot of supernatant was incubated with substrate at room temperature for 1 hour and the light intensity was measured using a Model 20e luminometer (Turner Designs, Sunnyvale, CA). The cotransfected luciferase activity of the extract was determined using a kit from Promega (Madison, WI). An aliquot of the supernatant was incubated with the luciferase substrate luciferin, and the light intensity emitted by the sample was measured using the luminometer. β-Gal activity was normalized to the cotransfected luciferase activity to standardize the efficiency of transient transfections. In some transfections, β-gal activity was normalized to cellular protein level, which was measured using the Bradford method [21].

X-gal In Situ Staining

Cells were fixed in 0.2% glutaraldehyde and 2% paraformaldehyde for 5 minutes, and washed twice with PBS. The cells were immersed in a staining solution containing 100 mM sodium phosphate (pH 7.3), 1.3 mM $MgCl_2$, 3 mM $K_3Fe(CN)_6$, 3 mM $K_4Fe(CN)_6$, and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal, 1 mg/ml) and incubated at 37°C for 18 hours. The stained cells were washed twice with PBS and examined under a light microscope.

Results

Induction of HIF-1α Expression under Anoxic Conditions in Glioblastoma Cell Lines

To determine whether HIF-1α expression was induced under anoxic conditions, w analyzed HIF-1α levels under both oxic and anoxic conditions in two glioblastoma cell lines using Western blots. After exposure to either oxic or anoxic conditions for 16 hours, cells were harvested and total protein was extracted from these cells. Equal amounts (25 μg) of protein were analyzed by 7% SDS–polyacrylamide gel and immunoblotting was performed using a monoclonal anti-human HIF-1α antibody. U-87 MG cells showed minimal expression of HIF-1α under oxia and a strong signal under anoxia; by densitometry measurement there was about a 15-fold increase in gene expression under anoxia (Figure 2). In U-251 MG cells, HIF-1α showed strong expression under oxia, and anoxia exposure increased expression only mildly (~1.4-fold) (Figure 2).

Effect of HRE Copy Number on Anoxia-Induced Gene Expression

More than a dozen hypoxia-regulated genes have been identified as containing HREs in their flanking regions [9]. In our study, we have used an HRE derived from the human Epo 3′ flanking region in its natural context. To investigate the influence of HRE copy number on the induction ratio of hypoxic to oxic heterologous gene expression in these cells, 3X, 6X, and 9XHRE were inserted adjacent to an SV40 minimal promoter in a mammalian expression vector that contained the *LacZ* gene (Figure 1). We have previously compared pH3LacZ gene expression under both oxic and anoxic conditions in U-251 MG and U-87 MG cells [17]. In the present paper, we first asked whether increasing HRE copy number from 3 to 6 or 9 would affect gene expression under oxic conditions. We transiently transfected these plasmids into U-87 MG and U-251 MG human glioblastoma cells using the lipofectamine-mediated method. To correct for variation in the efficiencies of transfection, we included in the DNA/liposome complex a control plasmid containing the luciferase reporter gene. Forty hours after transfection, we collected protein extracts from transfected cells and assayed them for β-gal activity, which was normalized by the cotransfected luciferase activity. As shown in Table 1, for U-251 MG cells, increasing HRE copy number above 3 resulted in very similar basal gene expression under oxic conditions. For U-87 MG cells, basal gene expression

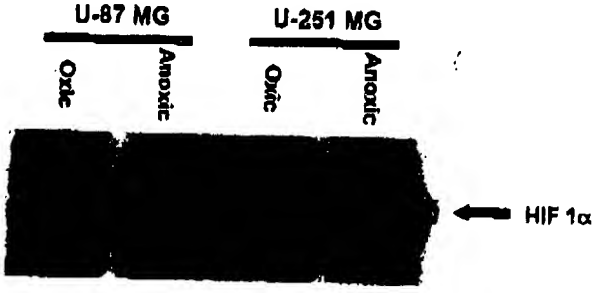

Figure 2. *Cell type–specific induction of HIF-1α under anoxic conditions. After exposure to either oxic or anoxic conditions for 16 hours, U-87 MG and U-251 MG cells were harvested and lysed. Equal amounts (25 μg) of proteins were analyzed by 7% SDS-PAGE and immunoblotting was performed using a monoclonal anti-human HIF-1α antibody (Novus Biologicals). U-87 MG cells showed minimal expression of HIF-1α under oxia and significantly increased HIF-1α under anoxia. In contrast, in U-251 MG cells HIF-1α showed considerable expression under oxic conditions and increased only slightly under anoxic conditions.*

Table 1. Gene Expression in Oxic Cells Exposed to Gene Constructs Containing *LacZ* under the Control of 3X, 6X, and 9XHREs.

Plasmid	Normalized β-gal Activity (relative light units/μg)*	
	U-251 MG	U-87 MG
pH3LacZ	23.3±7.0	28.7±5.1
pH6LacZ	29.9±2.0	22.3±3.7
pH9LacZ	28.8±2.8	45.2±8.5

*Numbers with errors in the table are the means and standard deviations of three independent samples.

remained similar for 3X and 6XHRE, however, for 9XHRE the average basal gene expression was about two-fold higher than for either 3X or 6XHRE (Table 1).

This increase in basal gene expression under oxic conditions, however, was very small compared to that observed under anoxic conditions. To measure this, we transiently transfected the plasmids into U-251 MG and U-87 MG cells. Twenty-four hours after transfection, cells were made anoxic for 16 hours. At the end of the anoxic period, we collected protein extracts from transfected cells and assayed them for β-gal activity. The β-gal activity in cells that were maintained under oxic conditions served as a control. In U-251 MG cells, 3XHRE increased gene expression about four-fold under anoxia (Figure 3), consistent with our previous findings [17]. Expanding HRE copy number to 6

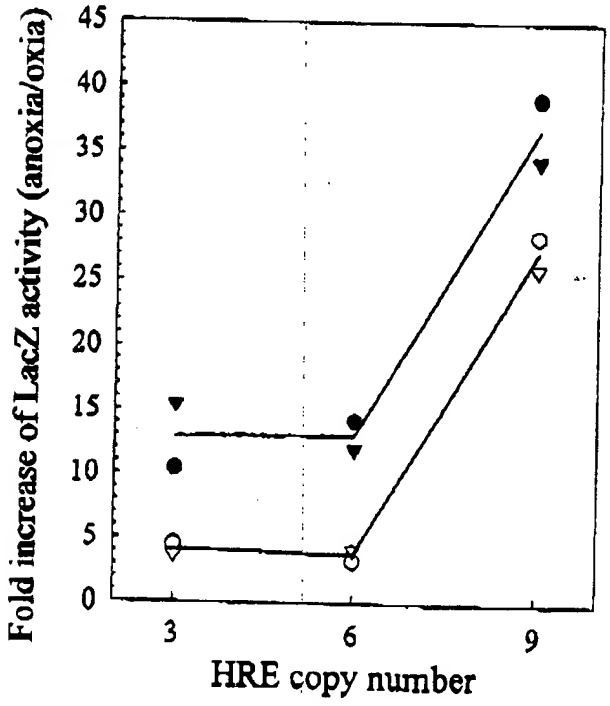

Figure 3. *Anoxia-induced LacZ expression depends on HRE copy number. U-87 MG and U-251 MG cells were transiently transfected with plasmids containing the LacZ reporter gene and 3X, 6X, and 9XHRE. Transfected cells were incubated under either anoxic or oxic conditions for 16 hours before analysis for LacZ activity. The relative level of LacZ expression in oxic cells was designated as 1.0. The actual values obtained from two independent experiments are shown for U-87 MG cells (−●−, −▼−) and U-251 MG cells (−○−, −▽−).*

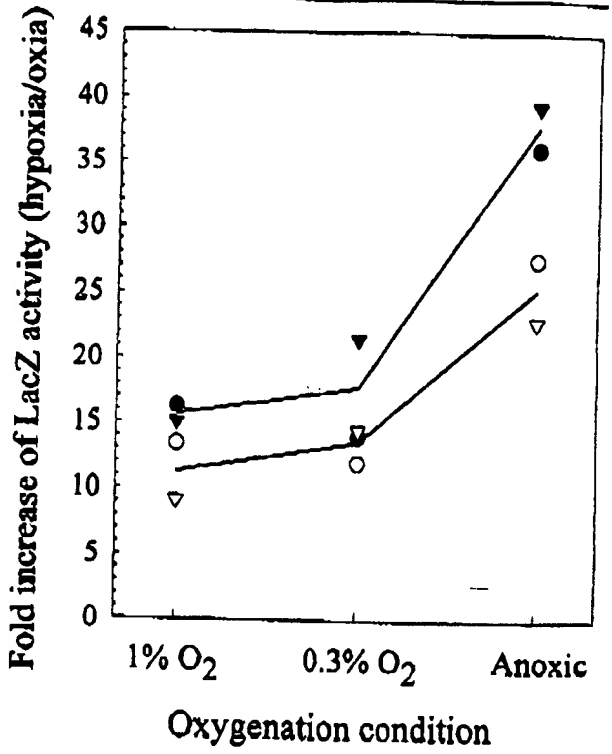

Figure 4. *Differential regulation of LacZ expression in pH9LacZ under intermediate oxygen concentrations. U-87 MG and U-251 MG cells were transiently transfected with the plasmid containing LacZ reporter gene and 9XHRE. Transfected cells were incubated under different oxygen concentrations for 16 hours before analysis for LacZ activity. The relative level of LacZ expression in oxic cells was designated as 1.0. The actual values obtained from two independent experiments are shown for U-87 MG cells (−●−, −▼−) and U-251 MG cells (−○−, −▽−).*

did not further increase gene expression compared with 3XHRE. However, when HRE copy number was increased to 9, we observed that gene expression increased to ~27-fold. We observed similar patterns of induced gene expression in U-87 MG cells; both 3X and 6XHRE produced about a 13-fold increase in gene expression, whereas 9XHRE further increased gene expression to ~37-fold (Figure 3).

Effect of 9XHRE on Gene Expression under Intermediate Oxygen Concentrations

Because many hypoxic tumor cells exist under intermediate oxygen levels [22], a successful hypoxia-targeted gene

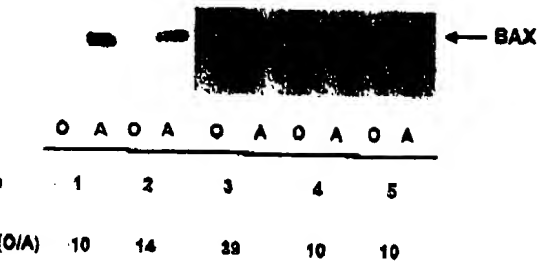

Figure 5. *Western blot showing BAX expression under Oxic (O) and Anoxic (A) conditions in pH9BAX–stably transfected U-251 MG clones. The intensity of the BAX protein was quantified using densitometry; the fold increase in BAX expression under anoxic conditions ranged from 10 to 39.*

Table 2. Infection of U-251 MG Cells by the recombinant AAV, AAVH9LacZ*.

Experiment	Normalized β-gal Activity† (relative light units/μg)	Fold of Increase (Anoxic/Oxic)
Oxic		
1	56.0 ± 2.7	
2	76.7 ± 15.5	
Anoxic		
1	6140 ± 786	110
2	6055 ± 548	79

*Multiplicity of infection (MOI) = 25.
†Numbers with errors in the table are the means and standard deviations of four independent samples.

therapy will need to be functional under a range of hypoxic conditions. Therefore, we determined whether 9XHRE could increase gene expression under two intermediate levels of hypoxia. For these studies, we transiently transfected pH9LacZ into both U-87 MG and U-251 MG cells. Transfected cells were then incubated under 1% oxygen, 0.3% oxygen or anoxic conditions. After a 16-hour incubation period, cells were harvested and β-gal activities were assayed. Compared to oxic conditions, gene expression in U-251 MG cells increased 11- and 14-fold under 1% and 0.3% oxygen, respectively, whereas the fold increase under anoxia was ~25 (Figure 4). Similarly, gene expression in U-87 MG cells increased 16- and 18-fold under 1% and 0.3% oxygen, respectively (Figure 4), whereas the fold increase under anoxia was ~38.

Evaluation of an HRE Vector that Expressed BAX Under Hypoxia

We demonstrated previously that overexpression of the pro-apoptotic BAX gene can kill anoxic tumor cells [17], indicating BAX might be a potentially useful suicide gene in hypoxia-targeted gene therapy. To determine whether the 9XHRE vector system could be employed to specifically express a suicide gene under hypoxic conditions, we generated pH9BAX. We then transfected this plasmid into U-251 MG cells and selected for stably transfected clones. Five clones were analyzed for BAX expression under both oxic and anoxic conditions using the Western blot method. As shown in Figure 5, under oxic conditions there was no detectable BAX expression in all five clones, indicating the 9XHRE regulatory system displayed minimal promoter leakage. When these clones were incubated under anoxic conditions for 16 hours, however, BAX expression was significantly upregulated in all clones. Densitometric scanning of the Western blots indicated that BAX expression increased from 10- to 39-fold, compared with the minimal expression measured under oxic conditions.

Hypoxic Induction in Glioblastoma Cells Infected with AAVH9LacZ Virus

To examine the magnitude of hypoxic induction in cells infected with the AAVH9LacZ virus, exponentially growing U-251 MG cells were infected with the recombinant virus. One day after infection, we incubated cells under anoxic or oxic conditions for 16 hours and then measured the levels of

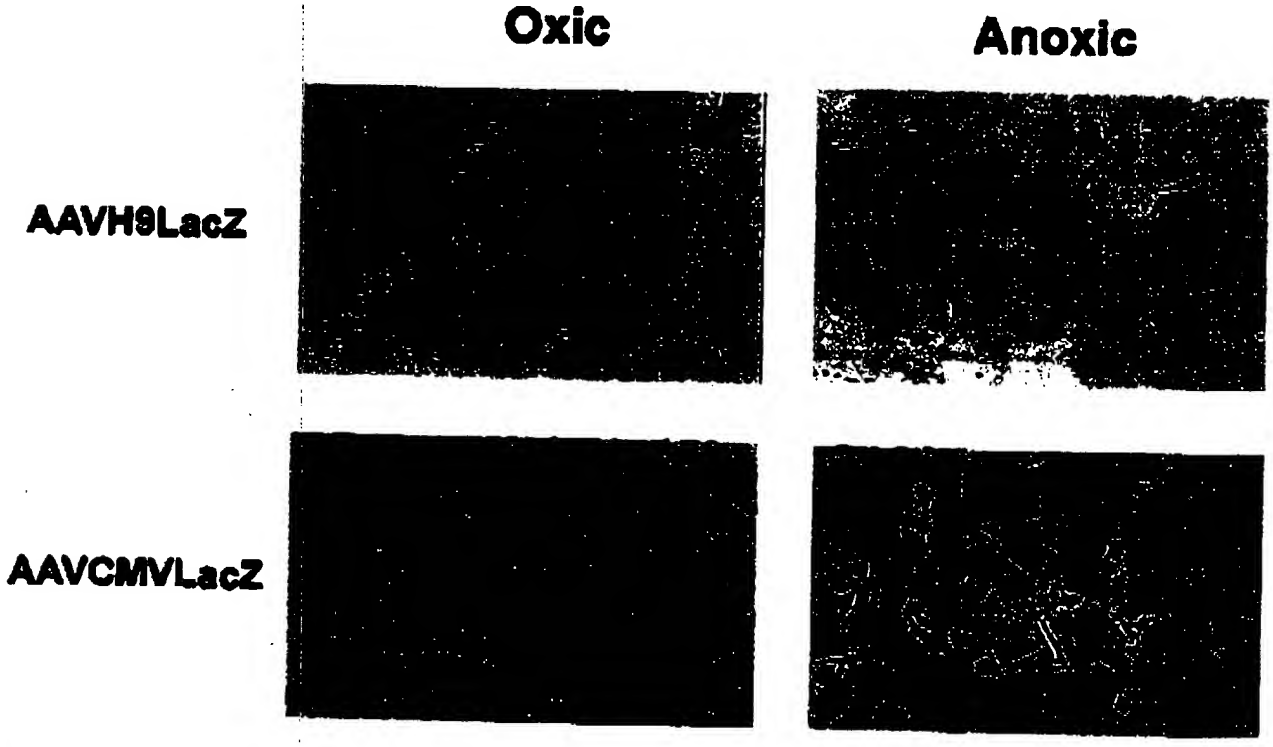

Figure 6. β-gal expression in U-251 MG cells. U-251 MG cells (3×10^5) were infected with either AAVH9LacZ or AAVCMVLacZ at an MOI of 25 and incubated under either oxic or anoxic condition for 16 hours. At the end of incubation, cells were assayed for β-gal expression by the X-gal in situ staining method as described in the Materials and Methods section.

β-gal quantitatively using a chemiluminescent assay. Significant increases of β-gal were detected in the anoxic cells compared with the oxic cells. As shown in Table 2, anoxic incubation gave 79- to 110-fold increase in gene expression compared with oxic conditions in two independent experiments. In addition, we found that the absolute level of gene expression from 9XHRE/Psv40 promoter was about 70% of that from the strong constitutive CMV promoter under anoxic conditions. Infected U-251 MG cells were also stained for β-gal expression using the X-gal *in situ* staining method. As shown in Figure 6, β-gal expression was minimal when cells were incubated under oxic conditions. However, anoxic incubation led to a significant increase in β-gal expression. As a control, β-gal expression from AAVCMVLacZ virus showed strong expression under both oxic and anoxic conditions.

Discussion

Hypoxia is a well-recognized feature of human brain tumors and is an important factor in tumor angiogenesis, invasion, and malignant growth [13]. A recent clinical study by Rampling and colleagues [3] made multiple measurements of pO_2 at various locations in primary and metastatic brain tumors. The mean pO_2 in tumors ranged from 0.01% to 3.2%, and the percentage of measurements that were <0.3% in each of these tumors ranged from 9.5 to 68.5%. Solid tumors cannot grow beyond a volume of several cubic millimeters in the absence of vascularization, and there is an inverse relation between tumor hypoxia and patient survival [9]. Overall, both basic and clinical studies have pointed out that tumor hypoxia is a significant problem for tumor resistance to radiotherapy and chemotherapy [4].

Our goal is to develop a gene therapy strategy to specifically kill hypoxic tumor cells. In combination with radiation therapy, which can efficiently kill well-oxygenated cells, this gene therapy approach should improve the therapeutic outcome for brain tumor patients.

HIF-1 is the master regulator of hypoxia-inducible gene expression and increased expression of HIF-1α protein has been detected in cancer cells. In this study, we first measured the expression of HIF-1α in two human glioblastoma cell lines. We found that HIF-1α protein in U-87 MG cells was minimal under oxic conditions but increased significantly when cells were incubated for 16 hours under anoxic conditions. In contrast, HIF-1α protein in U-251 MG cells was detected under both oxic and anoxic conditions, with only a slight increase in response to anoxic exposure. However, the absolute levels of gene expression under oxic conditions as measured by β-gal activities were essentially equivalent in both cell lines. We postulate that U-251 MG cells may have developed mechanisms to suppress activity of HIF-1 and render it nonfunctional under oxic conditions. It will be interesting to compare the expression levels of HIF-1 target genes such as VEGF between these two cell lines. These two cell lines are also heterogeneous with respect to other biologic properties. For example, these cells exhibit different sensitivities to anoxia; exposure of U-87 MG cells for 16 hours kills ~50% of the cells, whereas very few U-251 MG cells are killed under these conditions (Ruan and colleagues, unpublished data). Furthermore, it is known that U-87 MG cells carry a wild-type p53 gene, whereas p53 in U-251 MG cells is mutated [23]. Loss of wild-type p53 function in tumor cells has been implicated in enhancing HIF-1α levels in response to hypoxia [24]. Our data support the notion that the mutated p53 gene in U-251 MG cells might result in increased expression of HIF-1α even under oxic conditions.

Several studies by others indicate that the hypoxic environment can be used to activate heterologous gene expression driven by HRE. For example, Dachs and colleagues [25] showed that an HRE from the mouse phosphoglycerate kinase-1 gene could be used to control expression of marker and the therapeutic genes *in vitro* and within a solid tumor *in vivo*. In another study, Shibata and colleagues [26] have developed a hypoxia-responsive vector containing 5XHRE derived from the human VEGF gene combined with a CMV minimal promoter. Cells transfected with the vector exhibited over 500-fold increased transgene expression under hypoxia, similar to the level of the intact CMV promoter.

The amount of induced gene expression required to achieve selective tumor cell killing under hypoxia needs to be empirically determined for any given experimental system. Too low a ratio will not kill enough tumor cells, whereas too high a ratio may cause an adverse effect on surrounding normal cells. Therefore, in this study we investigated how increased gene expression under hypoxic conditions depends upon the HRE copy number. We chose the 31-bp human Epo HRE sequence for this study, and we investigated the hypoxia-induction ratio in plasmids containing 3X, 6X, and 9XHRE. Our results indicate that 9XHRE constructs produced the highest level of gene expression in both human glioblastoma cell lines studied. It remains to be determined whether increasing HRE copy number beyond 9 will further increase the hypoxia-induction ratio.

In our model systems, HRE copy number is an important component in the regulation of hypoxia-induced gene expression. Our data also indicate that the SV40 minimal promoter is a suitable basal promoter to be used in combination with HRE enhancers, because increased gene expression occurred under hypoxic conditions without any important increase in gene expression under oxic conditions (i.e., leakage). Boast and colleagues [27] also found that the SV40 minimal promoter and 3XHRE from the polyglycerate kinase gene gave a 146-fold induction of gene expression under hypoxia without increased basal level expression.

Because we wish to develop a therapy that kills tumor cells under hypoxic conditions, we have tested hypoxic induction of the suicide gene BAX. BAX is a member of the Bcl-2 protein family and overexpression of BAX causes apoptosis in both cell cultures and animals [28]. We have recently analyzed BAX expression in U-251 MG clones stably transfected with the construct containing 3XHRE and BAX [29]. Our results suggested that 3XHRE may be

insufficient to increase BAX expression under hypoxia in these clones. Because gene induction under hypoxia in the context of 9XHRE is significantly higher than that in 3XHRE, we decided to generate stable U-251 MG clones transfected with pH9BAX and examine hypoxic induction of BAX expression. There was minimal or und tectable BAX expression under oxic conditions in all clones, indicating no promoter leakage. Under anoxic conditions, we observed strong induction of BAX expression, ranging from 10- to 39-fold. Although we do not yet know how much increase in BAX gene expression is needed to kill hypoxic cells, studies on other therapeutic genes, such as cytosine deaminase and HSV-thymidine kinase genes, have found that only an approximately two- to seven-fold increase in gene expression was sufficient for hypoxic cell killing [25,30]. The magnitude of BAX expression needed to kill hypoxic cells in each of our model systems will have to be determined empirically.

Because it is unlikely that all tumor cells *in vivo* can be transfected with the suicide gene, the bystander effect will need to play a prominent role in successful gene therapy [31]. The development of the stably transfected pH9BAX clones will allow us to study any bystander effect that may be elicited by BAX. Ideally, the bystander effect will occur primarily in tumor cells and have limited effect on adjacent normal cells. It will be necessary to determine what the optimal level of BAX induction is to achieve such a beneficial bystander effect.

To exploit hypoxia-targeted gene therapy using an HRE-regulated system, it will be important to use an effective delivery system. Recently, an adenoviral vector, in which the transgene was under transcriptional control of HRE, produced inducible gene expression in response to hypoxia in a range of cell lines [32]. However, there are problems associated with adenovirus that could hamper its potential application for gene therapy in humans. These include the host immune reaction and inflammatory response elicited by viral genes and the reported toxicity of adenovirus to human tissues such as liver [33]. We decided to use an AAV vector system, because in the rAAV vector, both viral genes are removed and replaced with the desired therapeutic gene. Therefore, the risk of immune reaction is minimized because the vector cannot code for its own gene products [34]. Also, AAV is efficient in delivering its gene to both dividing and nondividing cells. This capability is important for the type of gene therapy that we propose, because most hypoxic cells are expected to be quiescent.

Finally, it will be critical that the AAV vector be able to reach tumor cells and it has to be minimally toxic to the surrounding normal brain tissue. The broad tropism of AAV makes targeting gene expression particularly important, and the goal for a targeted gene delivery system is to achieve low basal activity with high inducible expression levels. For example, hepatocarcinoma-specific expression of a therapeutic gene in rAAV has been demonstrated by employing the α-fetoprotein enhancer and albumin promoter; α-fetoprotein is expressed in most hepatocarcinomas with no detectable levels in normal adult liver tissue [35]. These tumor-specific parameters were also achieved in our rAAV, AAVH9LacZ, through the regulation of hypoxic conditions. When we inserted the DNA fragment containing 9XHRE and *LacZ* into the AAV vector, the resulting rAAV could be specifically regulated by hypoxia. *LacZ* expression was 79- and 110-fold higher under an xic conditions compared to oxic conditions in U-251 MG human glioblastoma cells. X-gal *in situ* staining experiments also confirmed increased gene expression in AAVH9LacZ-infected U-251 MG cells under anoxic conditions. Furthermore, the activity of the 9XHRE/Psv40 promoter is comparable with that of CMV promoter under anoxic conditions, indicating this promoter can lead to strong expression of the target gene. Similar HRE-regulated systems have been described previously. When an expression cassette containing 3XHRE derived from the human phosphoglycerate kinase gene linked with the SV40 minimal promoter was configured into an adenoviral vector, a range of cell types displayed low basal transgene expression and highly inducible level under hypoxia [32].

In summary, we have developed a novel rAAV in which transgene expression was specifically regulated by hypoxia, resulting in strong induction under hypoxic conditions. We believe this hypoxia-regulated rAAV vector will provide a useful delivery vehicle for tumor-targeted gene therapy, and we plan to test this hypothesis *in vivo*.

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